Spectrophotometer

Photometers and **spectrophotometers** are used to measure quantities such as absorbance and transmittance, but sometimes also turbidity. Devices that measure at one or only a few precisely defined wavelengths of monochromatic light are called **photometers**. Technically more complex and perfect instruments that allow the wavelength of monochromatic light to be set arbitrarily, or to measure a part of the absorption spectrum in a certain section of wavelengths, are called **spectrophotometers**.

Photometer Arrangement

In general, both photometers and spectrophotometers consist of four parts:

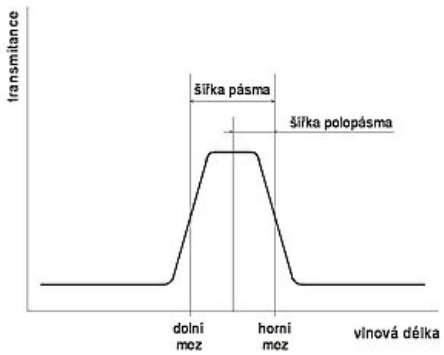
1. Light source
2. Monochromator
3. Compartment in which the sample is placed
4. Detector

Light source

A suitable light bulb or discharge lamp serves as a light source. Incandescent and halogen lamps provide continuous spectrum radiation in the visible and infrared regions, but cannot be used for measurements in the UV region. Hydrogen or deuterium discharge lamps are most often used as sources of ultraviolet radiation. The source of UV and visible light can also be, for example, a xenon discharge lamp, but the wide range of wavelengths is balanced by some disadvantages: its light is a composition of continuous and line spectrum, so there are large differences between the intensities at different wavelengths, the discharge lamp is very expensive and its intensity the light is not very stable.

Monochromator

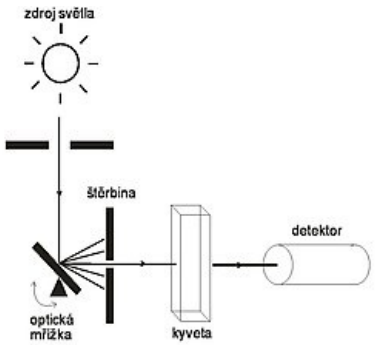
Polychromatic light then passes through a monochromator. The simplest and cheapest option is to include a suitable interference filter in the optical path. Filters for practically any wavelength in the ultraviolet and visible regions are commercially available today. There are several types of filters, the appropriate combination of which creates a filter with the required properties. Low-pass filters pass light of wavelengths shorter than a certain limit (cut-off). High-pass filters, on the other hand, only let in light that has a longer wavelength than the cutoff wavelength of the filter. Bandpass filters pass a certain range of wavelengths. Since the boundaries are not always sharp, the lower and upper limits are usually the wavelength for which the filter has a fifty percent transmittance compared to the wavelength it transmits best. Sometimes the center wavelength that the filter passes and the bandwidth (or half-bandwidth) are also given.



The bandwidth and half-band of the interference filter

Usually today, an optical grating serves as a monochromator, by tilting which the wavelength can be continuously changed (e.g. the so-called Czerného-Turner monochromator). The range of wavelengths that emerge from the monochromator is determined by the slit, either fixed or also adjustable.

The wider the slit, the greater the intensity of the outgoing light, but at the cost of less measurement specificity. On the other hand, a narrower slit will ensure more accurate adherence to the required wavelength, but at the cost of lower light intensity and deterioration of the signal-to-noise ratio.



Arrangement of a spectrophotometer

Sample

Monochromatic light passes through the sample. We mostly work with solutions that are filled into standard cuvettes with an optical path of 1 cm. The cuvettes are placed in the cuvette in the instrument, which ensures their exact position, can be tempered and sometimes also contains a magnetic stirrer, which can be used to mix the contents of the cuvette after inserting the stirrer into the cuvette during the measurement. It is often possible to insert several cuvettes into the cuvette at the same time, which are then automatically inserted into the optical path.

Detector

The light emerging from the sample finally hits a detector, usually a photodiode or other photoelectric element. The intensity is evaluated using a transducer system, compared to the intensity of light passing through the blank, and thus the absorbance is obtained. The accuracy of the measurement is affected by the integration time - the time for which the absorbance is measured. The longer it is, the more accurate the measurement result will be, unless, of course, the absorbing substance is photosensitive (ie, if the sample does not fade with longer exposure). The disadvantage of a long integration time is of course also the extension of the measurement time, which is essential especially when processing a large number of samples, when measuring at a large number of wavelengths (i.e. when measuring spectra), or when processing samples that change over time (kinetic measurements).

In addition to the so-called single-beam photometers, in which a blank sample is first measured and then the measured sample is inserted into the same optical path, so-called double-beam photometers are also used, which are equipped with two detectors and allow the blank and the measured sample to be measured simultaneously in two optical paths.

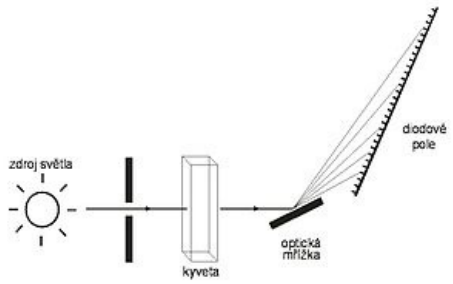
Other spectrophotometer arrangements

Measuring a spectrum on an optical grating spectrophotometer means that the instrument measures absorbance at one wavelength, then moves the grating, measures at another wavelength, and repeats this over and over until the entire desired area is measured. This entails a relatively long measurement time, which can be a drawback for several reasons:

- The sample can change over time (especially in kinetic measurements)
- The sample may be photosensitive, it fades during the measurement
- If multiple samples are processed that are not sufficiently stable, it may be technically difficult to ensure the same conditions for the first and last samples
- The measurement takes a long time, the performance of the method is low

Diode Array

The aforementioned shortcomings are eliminated by another arrangement of the spectrophotometer, measurement using a diode array (diode-array). In it, white light passes through the sample, which is then split into individual wavelengths (usually by means of a fixed optical grid) and falls on a plate with a large number of detectors - photodiodes (hence the name diode array). The diode array is positioned in such a way that a certain (relatively narrow – e.g. 2 nm) range of wavelengths falls on each photodiode. The device does not contain any moving elements, which increases the reproducibility of the measurement, and in addition, the entire spectrum is measured at once. The measurement time can thus be reduced from several minutes to fractions of a second. In addition, this arrangement makes it possible to construct devices approximately an order of magnitude more accurate than classic photometers, which also require practically no maintenance, calibration, etc. The fundamental disadvantage is the purchase price is many times higher, but with the reduction in the price of miniature electronic elements, the cost of producing a diode array decreases rapidly.



Arrangement of a diode array spectrophotometer

Vertical beam photometry

In routine use, the classic arrangement of photometry also has other disadvantages:

- Requires a large amount of sample
- It has low performance, making the processing of individual samples is laborious
- Cuvettes are expensive and difficult to maintain
- Spectrophotometers are expensive

These disadvantages are largely eliminated by measuring in microtitre plates using a photometer with a vertical beam (plate reader). Samples are filled into polystyrene plates with 96 wells, but there are also other formats (from 4 to 384 wells). Special aids are available for working with microtitre plates (multichannel pipettes, repeating pipettes, etc.), which significantly speed up sample preparation. Unlike classic photometers, in which the absorbance is measured with a horizontal beam and the optical path is given by the thickness of the cuvette, in this case the measurement takes place using a vertical beam and the length of the optical path depends on the height of the level in the well. If we add a certain amount of colorless solution to the sample, the concentration of the absorbing substance will decrease, but at the same time the level of the solution in the well will increase proportionally (the optical path will be extended) and the resulting absorbance will be the same. Conversely, if part of the colorless solvent evaporates from the sample, the optical path will be shortened, but the concentration of the absorbing substance will rise and the absorbance will not change again. It can be said that, unlike classical photometry, where the absorbance corresponds to the concentration of the absorbing substance in the solution, the absorbance when measured with a vertical beam depends on the quantity of the absorbing substance in the sample. For vertical beam photometry, a much smaller volume of sample is required – the wells are usually filled with only 100 to 300 µl of solution. Microtitre plates are disposable and, given the number of samples measured at

the same time, are significantly cheaper than plastic cuvettes. Measuring all 96 wells usually takes only a few seconds. Also, plate photometers tend to be relatively cheap, as a monochromator they usually use a set of interference filters. Vertical beam photometry is especially often used in immunochemistry, especially in the ELISA methodology (hence the extended name of the device ELISA-reader). For greater clarity, in vertical beam photometry, in addition to the term absorbance, the term optical density (OD - from English optical density) is used. At the same time, the relationship applies

$$OD = A / l$$

Where OD is the optical density, A is the absorbance, and l is the optical path length.

The optical density therefore depends only on the properties of the sample, but not on the length of the optical path along which the light passes through the sample.

Reflectance photometry

Another photometric technique is reflection photometry – a technique routinely used, for example, in clinical biochemistry within so-called dry chemistry. In dry chemistry, biological material is not processed in the usual way in test tubes or other containers, but is applied to a film impregnated with individual components of the reaction mixture. The result is a change in the color of the field, which can be quantified using a reflectance photometer - the loss of light of a certain wavelength reflected from the field is measured. As a light source, either LEDs emitting a suitable wavelength or incandescent sources with interference filters are used. To increase the sensitivity, the reflected light is focused on the detector by a convex mirror (the so-called Ulbricht sphere). A classic example of dry chemistry and reflection photometry is the examination of urine using diagnostic strips, however, due to its simplicity and speed with an accuracy comparable to classical methods, the number of applications is rapidly expanding.

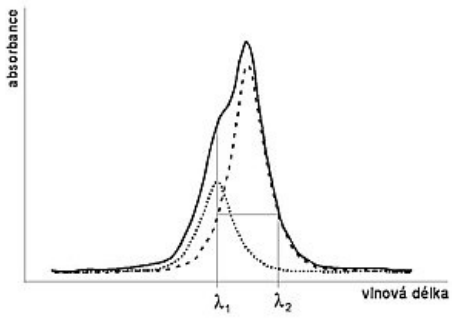
Advanced spectrophotometric techniques

Multicomponent Analysis

It often happens that in the part of the spectrum where the substance to be determined has an absorption maximum, another component of the reaction mixture also absorbs at the same time. In that case, it is not easy to determine the concentration of the measured substance, because the absorbance at the selected wavelength is the sum of the absorbances of both substances. If the concentration of the second, "interfering" component is known, or if the experiment can be set up so that it is constant in all samples, this situation can be resolved by using suitable blanks. In some cases, however, it is necessary to proceed with the so-called *multicomponent spectrum analysis methods*.

It is usually not measured at a single wavelength, but rather a continuous part of the spectrum is measured, or at least measured at several wavelengths. If the extinction coefficients of individual substances absorbing in a given region at different wavelengths are known (or even better, if the absorption spectra of individual components of the mixture are known), the concentration of the analyte can be calculated by solving a system of equations.

In the simplest case, it is necessary to determine the concentration of one substance, while its absorption spectrum (dotted in the figure) overlaps the spectrum of another substance present in the sample (dashed). By measuring the sample, we obtain a spectrum that is the sum of both absorption spectra (solid line). Even if we know that the measured substance has an absorption maximum at the wavelength λ1, we cannot determine its concentration directly, because at this wavelength the absorbance of the other substance cannot be neglected. However, if we know the shape of the absorption spectrum of the second substance, we can find the wavelength at which it absorbs the same as at the wavelength λ1 (let's denote it as λ2), i.e. the extinction coefficient is the same for λ1 and λ2. Then the absorbance of the determined substance itself will be.



Multicomponent analysis

$$A = A(\lambda_1) - A(\lambda_2).$$

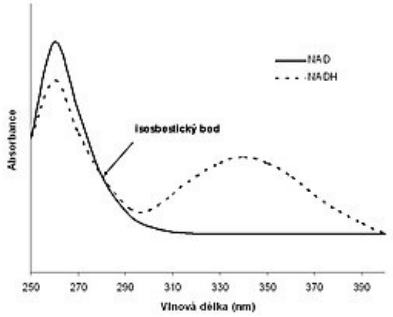
Use of the isosbestic point

If a substance that has a certain spectrum changes during some reaction to another substance that has a different spectrum, but the two spectra partially overlap. An example can be the spectra of NAD+ and NADH (the determination of these coenzymes is used when measuring the activity of a number of enzymes using the so-called Warburg optical test). In this case, the absorption maxima are far enough apart that the determination of the individual forms of the coenzyme is simple. However, the fact that all the spectra for different NAD+/NADPH ratios (at constant total concentration) cross over may be useful. This is due to the fact that at a wavelength of 281 nm both NAD+ and NADH have the same extinction coefficient. The intersection of the spectra is called the isosbestic point (from the Greek ισος isos = same and σβεννυμι sbennými = I extinguish) and by measuring the absorbance at this point we can easily determine the total concentration of NAD+ and NADH without needing to know the current ratio of the concentrations of both components.

Other methods

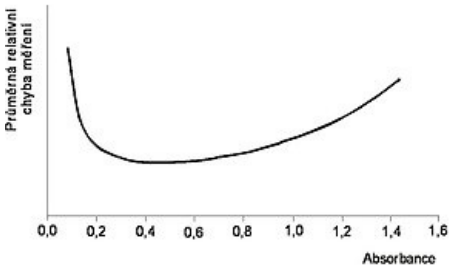
A number of other data can be obtained by appropriate mathematical processing of the measured spectra. When measuring more complex systems, derivative spectrophotometry can help, which allows, after evaluating the first and second derivatives of the spectra according to wavelength, for example to find the exact positions of absorption maxima, etc. Mathematical processing of the obtained data is also necessary for the processing of kinetic measurements.

Accuracy of photometric methods



Isosbestic point

Most photometers can measure absorbance in the range of 0 to 3 or 4. However, this does not mean that measuring over this entire range is reasonable. Assuming that the random error of the detector will always have the same properties, the average relative error of the absorbance measurement as a function of the actual absorbance of the sample will have a U-shaped curve. The best results will be obtained if the absorbance of the sample is in the range of 0.2 to 0 .8, measurements in the range of 0.2 to 1.2 can be considered reasonable. If the absorbance is higher, it is advisable to dilute the sample. Of course, the specific values depend on the properties of the photometer used, but the course of the average relative error as a function of absorbance will still be the same (only the "stretching" of the curve differs).



Average relative error of photometric determination

The accuracy of the measurement is also greatly influenced by the preparation of the sample and cuvette. Let us list at least the most significant influences that affect the accuracy of spectrophotometric determinations:

- must be measured in a suitable cuvette, the selected wavelength must lie in the band for which the cuvette is intended
- if measured in multiple cuvettes, they should all have the same factor. The plastic cuvettes should all be from the same batch.
- cuvettes for samples and blanks must be clean. This can be verified by filling them with distilled water all giving the same absorbance.
- the cuvettes must be handled in such a way as not to contaminate the optical surfaces (e.g. by touching the fingers). The same applies to microtitre plates.
- the cuvette must be dry on the outside, no air bubbles must remain inside, the measured solution must be homogeneous. A drop running down the cuvette, bubbles or a floating precipitate in the measured solution will usually show that the measured absorbance is constantly changing.
- the cuvette must be sufficiently filled with the sample.
- if several samples are measured successively in one cuvette, it is necessary to work in such a way that the error caused by the residues of the previous solution is as small as possible. Usually the cuvette is rinsed with distilled water between samples and then dried as best as possible. More accurate results can be obtained if, after washing, the cuvette is rinsed with a small amount of sample, which is poured out and only then is the cuvette filled with the required amount of sample for measurement. If working with several similar solutions, it may be more accurate not to rinse the cuvettes with distilled water between them, just pour them out and dry them as best as possible.
- when measuring in cuvettes, the concentration of the colored substance in the solution is essential, therefore, when preparing the sample, it is most important to observe the ratio of the individual components. It is advisable to arrange the experiment in such a way that all components are pipetted with the same pipette, preferably fixed.
- If it is measured in microtitre plates, on the other hand, the substance amount of the colored substance in the sample is decisive. Therefore, the experiment must be organized in such a way that the analyte is measured into the well as precisely as possible. A large error can also be caused by the different shape of the surface in the individual wells, therefore all samples should have the same affinity to the walls of the plate, or it is advisable to mix the plate briefly before the measurement to ensure that the walls are wetted even above the solution level.